Vitis **51** (4), 191–194 (2012)

Production of volatile compounds by mixed cultures of *Pichia guilliermondii* and *Saccharomyces cerevisiae*

G. Ciolfi, S. Favale and P. Pietromarchi

CRA - Unità di Ricerca per le Produzioni Enologiche dell'Italia Centrale, Velletri (Roma), Italy

Summary

During fermentation it was necessary to evaluate the united interactions in the metabolic activity of *Saccharomyces cervevisiae* x *Saccharomyces uvarum* (S6u) and *Pichia guilliermondii*, yeast which was present on the grapes in different proportions at the time of harvest.

The results highlight the fact that *Pichia guilliermondii* strongly influences the fermentation process and the total metabolic result.

For this reason, the presence of *Pichia guilliermondii* which is on the grapes at the time of harvest needs to be evaluated closely due to the fact it is competitive against *Saccharomyces* and it is important to note a velocity of sugar fermentation which is inferior to *Saccharomyces*.

For optimal regulation of fermentation it is necessary for the technician to detect *Pichia guilliermondi* presence in musts.

The massive presence of *Pichia guilliermondii* at pressing leads to slowing down or stoppage of fermentation and the production of high concentrations of benzyl alcohol and acetoin; it is therefore important to use healthy grapes at harvest and filtering the must if necessary to reduce the presence of *Pichia guilliermondii*.

K e y w o r d s : yeasts, *Saccharomyces cerevisiae*, *Pichia guillermondi*, wine fermentation.

Introduction

Alcoholic fermentation is a composite biochemical process involving the sequential development of various species of yeasts and bacteria. Yeasts are primarily responsible for the alcoholic fermentation of the grape juice. Wine can be produced by the natural fermentation by yeasts, that originate from grapes and winery equipment (Ribereau-Gayon *et al.* 2000). The perception of wine qualities is strongly related to the yeast activity in fermentation (Bisson and Karpel 2010). The various yeast species and strains that develop during the overall fermentation process metabolize grape juice constituents, principally the sugars, to a wide range of volatile and non-volatile end-products (Saez *et al.* 2010), which influence and determine the types and concentrations of many products that contribute to the aroma and flavor characteristics of the wine (Combina *et al.* 2005). Many ecological studies in different wine regions of the world have identified the main species of yeast that develop during fermentation (Jolly *et al.* 2003, Povhe-Jemec *et al.* 2001).

As is well known, interactions among yeasts and between yeasts and other microorganisms, present during fermentation, have been described as an additional stress factor that affects yeast growth in this substrate (Bauer and Pretorius 2000). Lopes *et al.* (2009) asserted that *Pichia guilliermondii* can produce volatile phenols similar to that produced by *Brettanomyces bruxellensis* (Barata *et al.* 2006, Martorell *et al.* 2006), at the initial stage of fermentation, and that yeast strains, isolated during fermentation, were very competitive. The authors (Lopes *et al.* 2009) studied the behavior of *Pichia guilliermondii* in different chemical and physical conditions in relation to pH, $S0_2$, temperature, alcohol, killer phenotype, but they did not analyze the metabolic behavior of *Pichia guilliermondii* during alcoholic fermentation, neither the interaction between metabolisms of *Pichia guilliermondii* and *Saccharomyces cerevisiae* x *uvarum*. The observations of these authors regarded the origin of the aroma "*brettanomyces*" in wine, which is not always exclusively due to the homonym species.

The present work started from the observation in some wine cellar where sluggish fermentation occurred. The experimental fermentation of musts obtained by unhealthy grapes harvested in the center of Italy (Frosinone), and thus treated near the harvest with commercial products containing copper, showed anomalies due to sluggish and slow fermentation which gave rise to excessive foam production, and a pungent and particular aroma.

That being so, the aim of this work was to verify the metabolic interactions during alcoholic fermentation between *Pichia guilliermondi*, yeast isolated in our laboratory, and a dried yeast *Saccharomyces cerevisiae* x *uvarum* for industrial use.

Material and Methods

The yeasts were *Pichia guilliermondii*, isolated during vinification with neutral character and identified (Kreger-van Rij, 1987), and S6u strain (*Saccharomyces cerevisiae* x *Saccharomyces uvarum*) dried yeast, hybrid with sensible character in comparison to the referal strain *Saccharomyces cerevisiae* (AT1) in our collection. The inoculation of

Correspondence to: Dr. G. Ciolfi, CRA - Unità di Ricerca per le Produzioni Enologiche dell'Italia Centrale, Via Cantina Sperimentale 1, 00049 Velletri (Roma), Italy. Fax. +39-069-634-020. E-mail: gaetano.ciolfi@entecra.it

$2x10^6$ cells·mL^{-1} of cells represents en experimental and conventional choice. Usually such a parameter reflects the level of programmed inoculation for a must but also the average load of indigenous flora present in the must.

The composition per liter of synthetic medium pH 3.20 was: $Na_2MoO_4 \cdot 2H_2O$ 200 µg; $ZnSO_4 \cdot 7H_2O$ 400 µg; $CuSO_4 \cdot 5H_2O$ 40 µg; H_3BO_3 500 µg; KI 100 µg; $FeCl_3 \cdot 6H_2O$ 400 µg; $MnSO_4 \cdot H_2O$ 400 µg; $NiCl_2 \cdot 6H_2O$ 400 µg; $K_2Cr_2O_7$ 20 µg; $CaCl_2$ 0.1 g; NaCl 0.1 g KH_2PO_4 1 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $(NH_4)_2SO_4$ 0.944 g; $(NH_4)_2HPO_4$ 0.943 g; tartaric acid 3 g; sucrose 200 g; KOH to pH 3.20; piroxydine hydrochloride 40 µg; thiamine hydrochloride 40 µg; myo-inositol 2 mg; biotin 20 µg; D-pantothenic acid calcium salt 400 µg; nicotinamide 40 µg; p-aminobenzoic acid 20 µg. Medium was sterilized by filtration with 0,2 µm membrane. The choice of this synthetic medium (Ciolfi et *al.* 1992) is due to reproducability motives because eventual interferences linked to the analytical variability of natural musts are eliminated. The trials, with relative inoculation percentages, have been named as:

Thesis 1: 100 % *Saccharomyces cerevisiae* x *Saccharomyces uvarum*; Thesis 2: 100 % *Pichia guilliermondii*; Thesis 3, 10 % *Saccharomyces cerevisiae* x *Saccharomyces uvarum* and 90 % *Pichia guilliermondii*; Thesis 4: 50 % *Saccharomyces cerevisiae* x *Saccharomyces uvarum* and 50 % *Pichia guilliermondii*; Thesis 5: 90 % *Saccharomyces cerevisiae* x *Saccharomyces uvarum* and 10 % *Pichia guilliermondii*. The fermentation was conducted in three repetitions in 1 L flasks filled with 800 mL of synthetic medium. The fermentation temperature was 20 °C.

During fermentation the development of alcohol was calculated by weight. The fermentation was interrupted when the drop in weight was lower than 0,05 % after three consecutive days. The analysis of volatile compounds was performed according to Giannotti and Di Stefano (2002). Twenty mL of wine were added with 200 µL of internal standard 1-heptanol (676 µg·L^{-1}), and diluted with 40 mL of H_2O prior to reverse solid phase extraction with C18 cartridges (1 g) and 6 mL of CH_2Cl_2 as eluent; dehydration of the extract, concentration under N_2 flow, and storing at -25 °C until analysis.

Gas chromatographic conditions: GC Fisons 8000 Mega series, autosampler, capillary column FFAP 50 m, id 0.32 mm widebore, 0.5 µm; pre-column 3 m; inj. 220 °C, det. 250 °C, He 2.9 mL·min^{-1}, split. 4.6, injection volume 3 µL; oven temperature: 33 °C 5 min, 3 °C·min^{-1} up to 220 °C, isothermal condition for 30 min. Identification and calibration was carried out in relation to commercial standards. Data were processed by analysis of variance (ANOVA) and Tukey's test with the software STATISTICA 7.1 (StatSoft, Italia 2005).

Results and Discussion

The trend of fermentation is shown in the Figure and the analytical results of the main enochemical parameters are shown in Tab. 1. The yeast population trend in mixed culture was observed daily. After 15 d of fermentation the yeast relationship was identical to that of inoculation. This shows how the reproductive speed of the two strains are almost identical. With respect to the fermentation delay, it appears that there is a slower metabolic activity when *Saccharomyces cerevisiae* x *Saccharomyces uvarum* (S6u) is associated in different proportions with *Pichia guilliermondii*.

In Tab. 2 the volatile compounds arising from metabolic activity are shown. When *Pichia guilliermondii* proportion increased in the medium of fermentation the competition with S6u is more evident, which results in a delay of the fermentation process. *Pichia guilliermondii* is characterized for the metabolic tendency to produce benzyl alcohol (bitter almond flavor) (Delfini *et al*. 1991) and acetoin (Benito *et al.* 2011) which are released in the medium.

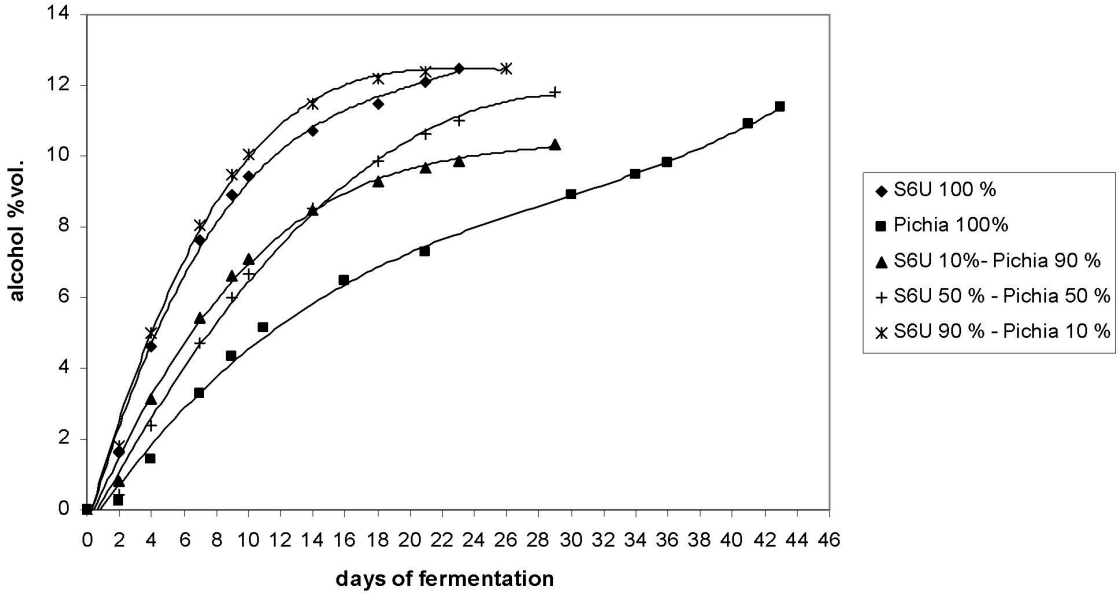

Figure: Trend of fermentations by pure and co-inoculated yeast strains *Saccharomyces cerevisiae x Saccharomyces uvarum* (S6u) and *Pichia guilliermondii.*

Table 1

Analysis of the main chemical parameters on wine after fermentation stopped

Chemical parameters	Thesis 1	Thesis 2	Thesis 3	Thesis 4	Thesis 5
Alcohol (% vol.)	12.50	11.39	10.33	11.82	12.50
Total reducing sugar ($g \cdot L^{-1}$)	n.d.	18.20	35.50	11.20	n.d.
Total acidity ($g \cdot L^{-1}$)	2.98	3.01	3.00	3.02	2.98
pH	3.19	3.20	3.20	3.18	3.21

The values presented are means of three replicates; n.d.= not detected

Table 2

Analysis of volatile compounds ($\mu g \cdot L^{-1}$) and statistical analysis (Test ANOVA, HSD Tukey)

Compound	Thesis 1 S6u 100 %	Thesis 2 *P. guilliermondii* 100 %	Thesis 3 S6u 10 % - *P. guilliermondii* 90 %	Thesis 4 S6u 50 % - *P. guilliermondii* 50 %	Thesis 5 S6u 90 % - *P. guilliermondii* 10 %
Isoamyl acetate***	$1060^{e} \pm 15.70$	$74^{a} \pm 9.10$	$236^{b} \pm 10.3$	$429^{c} \pm 15.10$	$511^{d} \pm 35.21$
Phenyl acetate***	$158^{d} \pm 9$	$20^{b} \pm 3.20$	$90^{c} \pm 6$	$125^{a} \pm 9.58$	$130^{a} \pm 8.79$
Σ Acetates***	$1218^{e} \pm 22.5$	$90^{a} \pm 13.5$	$326^{b} \pm 11$	$554^{c} \pm 18.50$	$641^{d} \pm 39.10$
Acetic acid ($g \cdot L^{-1}$) ***	$0.47^{a} \pm 0.02$	$0.80^{d} \pm 0.03$	$0.47^{a} \pm 0.02$	$0.42^{c} \pm 0.03$	$0.37^{b} \pm 0.01$
Σ (Acetates/acetic acid) x 10^3	2.59	0.11	0.69	1.32	1.73
Ethyl hexanoate***	$149^{a} \pm 10.33$	$70^{c} \pm 9.5$	$139^{a} \pm 11$	$129^{a,b} \pm 8$	$110^{b} \pm 5.40$
Ethyl octanoate***	$75^{b} \pm 5.01$	$21^{a} \pm 3.5$	$19^{a} \pm 3.50$	$20^{a} \pm 2.50$	$21^{a} \pm 1.60$
Ethyl decanoate***	$21^{c} \pm 3$	$3^{a} \pm 1.8$	$35^{d} \pm 4.20$	$15^{b,c} \pm 3.50$	$11^{a,b} \pm 2.80$
Σ Ethyl ester***	$245^{d} \pm 26.81$	$94^{c} \pm 15.2$	$193^{b} \pm 9.50$	$164^{a,b} \pm 9.40$	$142^{a} \pm 8.20$
Hexanoic acid***	$1782^{e} \pm 19.1$	$295^{a} \pm 15.01$	$1453^{d} \pm 16.50$	$1320^{c} \pm 35$	$1088^{b} \pm 26.87$
Octanoic acid***	$2520^{c} \pm 31.03$	$518^{b} \pm 12.04$	$2070^{a} \pm 17.30$	$2067^{a} \pm 29.70$	$2030^{a} \pm 28.91$
Decanoic acid***	$1258^{d} \pm 20$	$498^{b} \pm 10.30$	$1130^{a} \pm 13.23$	$1100^{a} \pm 25.40$	$1002^{c} \pm 13.33$
Σ Fatty acids***	$5559^{e} \pm 69.10$	$1311^{a} \pm 58.40$	$4653^{d} \pm 21.41$	$4487^{c} \pm 50.10$	$4120^{b} \pm 33.10$
Σ Ethyl ester / fatty acid	0.044	0.072	0.041	0.036	0.034
Butanoic acid***	$47^{b} \pm 3.20$	n.d.	$61^{c} \pm 7.23$	$38^{a,b} \pm 5$	$31^{a} \pm 6.11$
Dodecanoic acid***	$378^{b} \pm 15.8$	n.d.	$118^{a} \pm 18.8$	n.d.	n.d.
Isoamyl alcohol***	$53700^{d} \pm 15.30$	$12160^{a} \pm 62.5$	$56168^{e} \pm 71.3$	$52069^{c} \pm 65.1$	$46187^{b} \pm 71.11$
Benzyl alcohol***	n.d.	$158^{c} \pm 10$	$18^{b} \pm 5.16$	$12^{a,b} \pm 4.35$	$10^{a,b} \pm 2.33$
2-Phenylethanol***	$11951^{c} \pm 53.50$	$8327^{a} \pm 43.5$	$18487^{e} \pm 60.30$	$14335^{d} \pm 43.10$	$10831^{b} \pm 63.11$
Acetoin***	$28^{a,b} \pm 5.4$	$146^{c} \pm 11$	$44^{b} \pm 5$	$18^{a} \pm 3.11$	$15^{a} \pm 3.49$

Note: *** Highly statistical significant differences $\alpha = 0.05$. Data correspond to the means of three replication ± standard deviation. nd = not detected. Values with the same letter belong to the same group.

The benzyl alcohol comes from the biosynthetic pathway of phenols. That being so, the presence of benzyl alcohol in a medium of fermentation could be due to the presence of *Pichia guilliermondii*, this could justify how in some areas its incidence is particularly significant and this could point out how the author (Calò *et al.* 2006) finds a correlation between benzyl alcohol and 2-phenylethanol. From studies already carried out (Ciolfi *et al*. 1992), S6u results as being a good producer of 2-phenylethanol and the precursors of this compound including shikimic acid. This justifies how the quantity of 2-phenylethanol is greater when *Pichia guilliermondii* is associated with S6u because it takes advantage of the precursors produced by the latter (S6u).

In absolute terms, the biosynthesis of volatile compounds by *Pichia guilliermondii* appear very limited with respect to *Saccharomyces cerevisiae*, on the other hand the production of acetic acid appears to be high. In relation to the production of acetates, the esterification index increases when the percentage of *Saccharomyces* increased in the initial inoculation. The fatty acids C6, C8, C10 result as being less in the medium where *Pichia guilliermondii* was inoculated, but the esterification index was highest where this yeast was purely inoculated. The high synthesis of acetoin by *Pichia guilliermondii* secreted in the fermentation medium confers a typical tasting characteristic. The acetic acid deserves in-depth examination.

The statistical analysis applied to the volatile compounds highlights how the two theories have a significantly high comparability difference. It is interesting to note how the ethylC8 compound, in all the theories in which *Pichia guilliermondii is present,* assumes a relatively low value. This leads us to believe that *Pichia guilliermondii* could use the compound (ethylC8) for its own cellular metabolism.

Conclusion

The analysis of the metabolic activity of *Saccharomyces cerevisiae* x *Saccharomyces uvarum* (S6u) and *Pichia guilliermondii*, shows that the presence of *Pichia guilliermodii* strongly influence the trend of the alcoholic fermentation and the value of the metabolites in medium of fermentation. In the trial where *Pichia guilliermondii* fermented alone, there is a production of benzyl alcohol and acetoin much less noble in terms of flavor. With the above mentioned in mind, it is convenient to guarantee perfect grape health during the harvest and if this is not possible then to carry out microbiological analysis of the must. If there is a considerable presence of *Pichia guilliermondii* it could be possible to filter the must to bring down the level of the yeast and follow up with inoculation of selected yeasts.

References

Barata, A.; Nobre, A.; Correia, P.; Malfeito-Ferreira, M.; Loureiro, V.; 2006: Growth and 4-ethylphenol production by the yeast *Pichia guilliermondii* in grape juices. Am. J. Enol. Vitic. **57,** 133-138.

Bauer, F. F.; Pretorius, I. S.; 2000: Yeast stress response and fermentation efficiency: how to survive the making of wine - a review. S. Afr. J. Enol.Vitic. **21,** 27-51.

Benito, S.; Morata, A; Palomero, F; González, M. C.; Suárez-Lepe, J. A.; 2011: Formation of vinylphenolic pyranoanthocyanins by *Saccharomyces cerevisiae* and *Pichia guillermondii* in red wines produced following different fermentation strategies. Food Chem. **124**, 15-23

Bisson L. F.; Karpel, E. J.; 2010: Genetics of Yeast Impacting Wine Quality. Annu. Rev. Food Sci. Technol. **1**, 139-62.

Calò, A.; Tommasi, D.; Pascarella, G.; 2006: Le vigne del Tarantino. Quad. Vitic. Enol. Univ. Torino, Italy.

Ciolfi, G.; Garofolo, A.; Moretti, S.; Spera, G.; Morassut, M.; Cecchini, F.; 1992. Variazioni metaboliche del lievito in funzione delle differenti condizioni nutrizionali. Biologia Oggi, **1-2**, 127-144.

Combina, M.; Elía, A.; Mercato, L.; Catania, C.; Ganga, A.; Martinez, C.; 2005: Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. Int. J. Food Microbiol **99**, 237-243

Delfini, C.; Gaia, P.; Bardi, L.; Mariscalco, G.; Contiero, M. and Pagliara, A.; 1991: Production of benzaldehyde, benzyl alcohol and benzoic acid by yeasts and *Botrytis cinerea* isolated from grape musts and wines.Vitis **30**, 253-263

Giannotti, S.; Di Stefano, R.; 1991: Metodo per la determinazione dei composti volatili di fermentazione. L'Enotecnico. **10**, 61-64

Jensen, S. L.; Umiker, N. L.; Arneborg, N.; Edwards, C. G.; 2009: Identification and characterization of *Dekkera bruxellensis*, *Candida pararugosa*, and *Pichia guilliermondii* isolated from commercial red wines. Food Microbiol. **26**, 915-921.

Jolly, N.; Augustyn, O.; Pretorius, I.; 2003: The occurrence of non-Saccharomyces yeast species over three vintages on four vineyards and grape musts from four production regions of the Western Cape, South Africa. S. Afr. J. Enol. Vitic. **24,** 35-42.

Kreger-van Rij, N. J. W.; 1987: The Yeasts, a Taxonomic Study. Elsevier Science Publishers B.V. Amsterdam.

Lopes, C. A.; Sàez, J. S.; Sangorrìn, M. P.; 2009: Differential response of *Pichia guilliermondii* spoilage isolates to biological and physicochemical factors prevailing in Patagonian wine fermentations. Can. J. Microbiol. **55,** 801-809.

Martorell, P.; Barata, A.; Malfeito-Ferreira, M.; Fernandez-Espinar, M. T.; Loureiro, V.; Querol, A.; 2006: Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guilliermondii* recovered from wine related sources. Int. J. Food Microbiol. **106**, 79-84

Povhe-Jemec, K.; Cadez, N.; Zagorc, T.; Bubic, V.; Zupec, A.; Raspor, P.; 2001: Yeast population dynamics in five spontaneous fermentations of Malvasia must. Food Microbiol. **18,** 241-259.

Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud, A.; 2000: Handbook of Enology, the Microbiology of Wine and Vinifications. John Wiley and Sons (Eds), West Sussex (England).

Saez, J. S.; Lopes, C. A.; Kirs, V. C.; Snagorrin, M. P.; 2010: Enhanced volatile phenols in wine fermented with *Saccharomyces cerevisiae* and spoiled with *Pichia guilliermondii* and *Dekkera bruxellensis*. Lett. Appl. Microbiol. **51**, 170-176.

StatSoft Italia; 2005: STATISTICA (sistema software di analisi dei dati), vers.7.1. www.statsoft.it

Received November 15, 2011